EuPA OPEN PROTEOMICS 7 (2015) 54–63

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: http://www.elsevier.com/locate/euprot

Low molecular weight peptides derived from sarcoplasmic proteins produced by an autochthonous starter culture in a beaker sausage model

Constanza M. López[a], *Miguel A. Sentandreu*[b], *Graciela M. Vignolo*[a], *Silvina G. Fadda*[a,*]

[a] *CERELA – Centro de Referencia para Lactobacilos, CONICET, San Miguel de Tucumán, Argentina*
[b] *Instituto de Agroquímica y Tecnología de Alimentos (CSIC), Valencia, Spain*

ARTICLE INFO

Article history:
Received 3 October 2014
Received in revised form 2 May 2015
Accepted 5 May 2015
Available online 12 May 2015

Keywords:
Meat proteolysis
Lactic acid bacteria
Starter cultures
Peptidomics

ABSTRACT

This study focuses on meat protein degradation by different starter cultures. Sausage models inoculated with *Lactobacillus curvatus* CRL705 and *Staphylococcus vitulinus* GV318 alone and as a mixture were incubated 10 days at 22 °C. Low molecular weight peptides (<3 kDa) derived from sarcoplasmic proteins were analyzed by a peptidomic approach. A diverse number of protein fragments were identified. The greatest peptides diversity was obtained when the mixed starter culture was present. Peptides mainly arose from myoglobin, creatine-kinase, glyceraldehyde-3-phosphate-dehydrogenase and fructose-biphosphate-aldolase (ALDOA). ALDOA hydrolysis was attributed to the mixed starter culture; the released peptides could act as biomarkers for a specific sausage technology.

Significance: The selection of a specific autochthonous starter culture guarantees the hygiene and typicity of fermented sausages. The identification of new peptides as well as new target proteins by means of peptidomics represents a significant step toward the elucidation of the role of microorganisms in meat proteolysis. Moreover, these peptides may be further used as biomarkers capable to certify the use of the applied autochthonous starter culture described here.

1. Introduction

Cured products represent an important part of processed meat products. In particular, the manufacture of dry sausages has a long history in Argentina mainly owing to Italian and Spanish traditions as well as the well-known quality of beef meat. Fermented dry sausages (*salame*) are produced in different regions of Argentina using local artisanal techniques for their preparation, which includes beef and/or pork meat [1–3]. Although typical preparations based on indigenous microbiota still exist, the adequate selection of autochthonous starter cultures constitutes an effective tool toward a standardized process while preserving the original and typical

* *Corresponding author at*: CERELA, Chacabuco 145, T4000ILC Tucumán, Argentina. Tel.: +54 381 4311720x116; fax: +54 381 4005600.
E-mail address: sfadda@cerela.org.ar (S.G. Fadda).

http://dx.doi.org/10.1016/j.euprot.2015.05.001

sensory quality of the products [4–8]. Besides the high acidogenic competitiveness of starter strains to decrease pH, activities that improve flavor, color and hygienic safety should be targeted in the search for new strains. Concerning food safety, inactivation of pathogens and contaminants through acid production and bacteriocins; antibiotic susceptibility and the inability to produce biogenic amines are major traits to be considered [9]. Lactic acid bacteria (LAB) and Coagulase Negative *Staphylococci* (CNS) are the most important microbial groups used for starter culture formulations for meat products. While LAB acidify meat matrix, guaranteeing safety and typical sensory properties, CNS display both nitrate-reductase and catalase activities, contributing to the development of typical cured meat color [10].

In addition to acid production by LAB, starter strains exert proteolytic and lipolytic activities during sausage fermentation and ripening that play a key role to assure final specific sensorial characteristics [11,12]. LAB are endowed with proteolytic activity, mainly intracellular amino-, di- and tripeptidyl-peptidase activities [3]. The intracellular enzymes from *Lactobacillus sakei*, *Lactobacillus curvatus* and *Lactobacillus plantarum* were reported to be responsible for the generation of small peptides and amino acids [20–24]. Staphylococci also contribute to breakdown of meat proteins; particularly *Staphylococcus carnosus* and *Staphylococcus simulans* have been described to degrade sarcoplasmic proteins, while *Staphylococcus xylosus* was able to hydrolyze both, sarcoplasmic and myofibrillar proteins [19]. According to current knowledge, the meat protein degradation model involves the sequential action of endopeptidases and exopeptidases; proteins are first degraded by endopeptidases and the resulting fragments are further hydrolyzed by several types of exopeptidases such as amino- and carboxy-peptidases [13]. Proteolysis of dry cured meat products has been attributed to either endogenous enzymes [14,15] and/or exogenous enzymes from microorganisms [3,16]. However, the proteolysis pattern in fermented sausages is influenced by different variables such as product formulation, processing conditions and starter cultures [17].

Even if undeniable efforts to elucidate the proteolytic action of starter cultures during meat fermentation have been performed, the identity of the generated peptides from meat fermentation processes remains elusive. Recently, valuable biomarkers to monitor and predict meat quality have been identified by proteomic approaches [25]. Unlike proteomics, the novel concept of peptidomics aims at the comprehensive visualization and systematic analysis of small peptides content within an organism, tissue or cell (peptidome) in order to identify structure and function [26]. In fact, the study and exploration of complex peptidomes in food science have been undertaken by means of these global profiling approaches. These strategies have been applied in order to identify relevant peptides (bioactive peptides) as well as the analysis of peptide evolution, for example in Parmigiano-Reggiano cheese [27,28]. In addition, peptide fractions originated from meat have been identified as biomarkers for meat tenderness, authenticity and sensory attributes [29–32]. To the best of our knowledge, the identification of specific peptide sequences arisen from proteolysis in fermented sausages has not yet been accomplished.

On this basis, the aim of this study was to evaluate the effect of a selected autochthonous starter culture (*L. curvatus* CRL705 and *S. vitulinus* GV318) on sarcoplasmic protein hydrolysis during sausage processing by a peptidomic approach. The analysis of low molecular weight (LMW) peptides (<3 kDa) derived from sarcoplasmic proteins was assessed by Liquid Chromatography coupled to Electrospray Ionization tandem Mass Spectrometry (LC–MS/MS). This could provide not only a deeper understanding of meat proteolysis but also identify peptides with biomarker potential for technological/authentication purposes.

2. Materials and methods

2.1. Strains and culture conditions

L. curvatus CRL705, isolated from an artisanal fermented sausage (Tucumán, Argentina) was used based on its known technological and safety features [33–36]. It was routinely grown in de Mann Rogosa Sharpe (MRS) broth (Britania, Argentina) for 48 h at 30 °C. On the other hand, the Coagulase Negative *Staphylococcus* (CNS) to be used in the mixed starter culture was selected among strains previously isolated from different artisanal dry fermented sausages [1,37]. They were cultured in aerobiosis in Brain Heart Infusion (BHI) broth (Britania, Argentina) for 48 h at 37 °C and 150 rpm. Compatibility of seven CNS strains with *L. curvatus* CRL705 was also carried out by using the spot method [38].

2.2. Selection of CNS for mixed starter culture formulation

2.2.1. Antibiotic susceptibility assay

Susceptibility to antibiotics of seven CNS isolates was determined by the agar overlay disk diffusion test as recommended by the National Committee for Clinical Laboratory Standard (NCCLS, 2002). The antibiotics used for the test were: oxacillin (1 μg), penicillin G (10 U), erythromycin (15 μg), ciprofloxacin (5 μg), clindamycin (2 μg), tetracycline (30 μg), streptomycin (300 μg), neomycin (30 μg), novobiocin (5 μg), cefoxitin (10 μg), rifampicin (5 μg), vancomycin (30 μg), chloramphenicol (30 μg) and trimethoprim sulfametoxazol (5 μg) (Becton Dickinson).

2.2.2. Production of biogenic amines

The ability of CNS isolates to produce biogenic amines by decarboxylation of precursors amino acids was qualitatively tested [39]. To detect decarboxylating strains Joosten and Northolt medium modified by Fadda et al. [39] was used added with 2% L-histidine and L-tyrosine (Anedra, Germany). Plates with the agar medium were spotted with active strains and incubated at 30 °C for 2–5 days. Decarboxylating strains were easily recognizable by formation of a purple halo in the yellow medium.

2.2.3. Genotypic characterization of selected CNS isolates

The variable V1 region of 16s rDNA was sequenced at the Sequencing Service (CERELA). Sequences were analyzed by using BLAST (The Basic Local Alignment Search Tool) from

NCBI (http://www.ncbi.nlm.nih.gov/BLAST) and the Ribosomal Database (http://rdp.cme.msu.edu/).

2.3. Beaker sausage models (BS)

All operations were carried out in a laminar flow hood. Beef meat was obtained 24 h post-mortem from cooled carcasses, frozen and aseptically sampled by superficial burning, followed by removal of surface cuts using sterile knives and minced under aseptic conditions. One kg of aseptic minced meat was thoroughly mixed with the curing additives: 3% NaCl, 0.02% $NaNO_2$, 0.75% sucrose and 0.75% glucose, previously filtered-sterilized (0.22 μm) (Millipore, Billerica, USA). The meat components, curing salts and other ingredients were thoroughly mixed and the sausage batter was then divided into four portions: (i) BS-control was added with antibiotics (20.000 UI/kg penicillin, 20 mg/kg streptomycin and 50 mg/kg amphotericin B) (Gibco, Grand Island, USA); (ii) BS-Lc inoculated with *L. curvatus* CRL705 (7–8 log CFU/g); (iii) BS-Sv inoculated with *S. vitulinus* GV318 (6–7 log CFU/g) and (iv) BS-mix inoculated with both strains (7 and 6 log CFU/g for *L. curvatus* and *S. vitulinus*, respectively). Flasks containing the different BS models were incubated at 22 °C and samples were collected at 0, 1, 3, 10 and 15 days of fermentation and ripening. Three independent replicates were performed for each BS model.

2.4. Bacterial counts and pH measurement

Ten grams of each BS were mixed with 90 ml of sterile 0.1% peptone water (Britania, Argentine) and homogenized for 8 min in a stomacher machine (Lab Blender 400, Seward Medical, London, UK). Appropriate decimal dilutions of samples were prepared and plated in duplicate onto selective media for bacterial enumeration. Plate Count Agar and MRS agar (Britania, Argentine) were used for total aerobic mesophilic microorganisms and LAB, respectively and incubated at 30 °C during 48 h, while Mannitol Salt Agar was used for CNS (37 °C for 48 h). The pH values were obtained by directly inserting the tip of the probe (Meat pH Meter, Hanna Instruments, Argentina) into different portions of BS samples.

2.5. Peptide extraction

Ten grams from each BS sample were homogenized in stomacher during 8 min with 0.1 N HCl (1:5, w/v) according to Sentandreu et al. [30]. The meat slurries were centrifuged (13,500 rpm at 4 °C for 20 min) and supernatants submitted to ultra-filtration in Amicon Ultra-4 Centrifugal Filter Unit with Ultracel-3 membrane (Millipore, Billerica, USA). The obtained filtrate (approximately 3 ml) containing peptides lower than 3 kDa was frozen at –20 °C during 24 h and then freeze-dried until further LC–ESI-MS/MS analyses.

2.6. Peptide sequence identification by LC MS/MS

Freeze-dried samples corresponding to peptides lower that 3 kDa were re-dissolved in 1 ml of 0.1% trifluoroacetic acid (TFA). Twenty-five microliters of this solution were injected into a Surveyor LC system directly coupled to a LCQ Advantage Ion trap MS instrument (Thermo Scientific, San Jose, CA). Separation of peptides was carried out on a Jupiter Proteo reverse phase column (150 mm × 0.5 mm; Phenomenex, Torrance, CA), using the following conditions: linear gradient from 0 to 40% acetonitrile in 0.1% formic acid for 140 min at a flow rate of 30 μl/min. Operating parameters of the ion trap detector were the following: electrospray ionization in the positive mode, capillary temperature 250 °C, collision energy normalized to 35%, spray voltage 4.5 kV, and capillary voltage 33.0 V. First scan event was full MS detection for *m/z* values in the range 400–2000. The second event was a dependent MS/MS scan of the most intense ions having charges from +2 to +4, enabling dynamic exclusion after three scans of the most intense ion for a period of 5 min. The minimum ion intensity for triggering a MS/MS scan was 5×10^5. Data acquisition was done using the Xcalibur v2.0 software. Peptide identification was done from the information contained in the generated MS/MS spectral data using an in-house version of the Mascot search engine v2.3 (www.matrixscience.com) against the Uniprot KB protein database (www.uniprot.org). We defined the following search parameters: Enzyme: "none"; no fixed modifications, variable modifications: "Deamidation (NQ)" and "Oxidation (M)". Mass accuracy was set to 1.2 and 0.6 Da for MS and MS/MS mode, respectively. The option "Mammalia" was selected as taxonomy restriction parameter. For selection of the MS/MS identifications obtained with Mascot, only top ranking significant peptides were considered, taking a reference peptide score threshold of 25 for considering a true sequence. Selected identifications following these criteria were further verified by manually checking the assignation of the identified masses to *b* and *y* series ions. Peptide identifications were not considered in those cases where there was not a good correlative identification of several *b* and/or *y* ions (at least in part of the whole peptide sequence) or in those cases in which the MS/MS spectrum was of poor quality (too low peak information or too low signal to noise ratio).

3. Results

3.1. Selection of CNS for starter formulation

When compatibility of the seven CNS strains with *L. curvatus* CRL705 was performed using the plate assay, four strains (GV310, GV318, GV410 and GV718) were able to grow in the presence of *L. curvatus*, since no inhibition halo was observed. Then, antibiotic susceptibility test was assayed against 14 antibiotics of clinical relevance to these compatible CNS strains. Although double and single resistances patterns were obtained, GV318 displayed susceptibility to all assayed antibiotics. In addition, strain GV318 was not able to produce biogenic amines from the used precursor amino acids (data no shown). Based on these features, this strain was selected to complement *L. curvatus* CRL 705 in the mixed starter culture. Then, GV318 was subjected to a genotypic identification. After amplification of V1 variable region of 16s rDNA, sequences were compared with those in the NCBI and Ribosomal database, the results indicated high identity (99%) with *S. vitulinus*, confirming the identity of the selected isolate as *S. vitulinus* GV318.

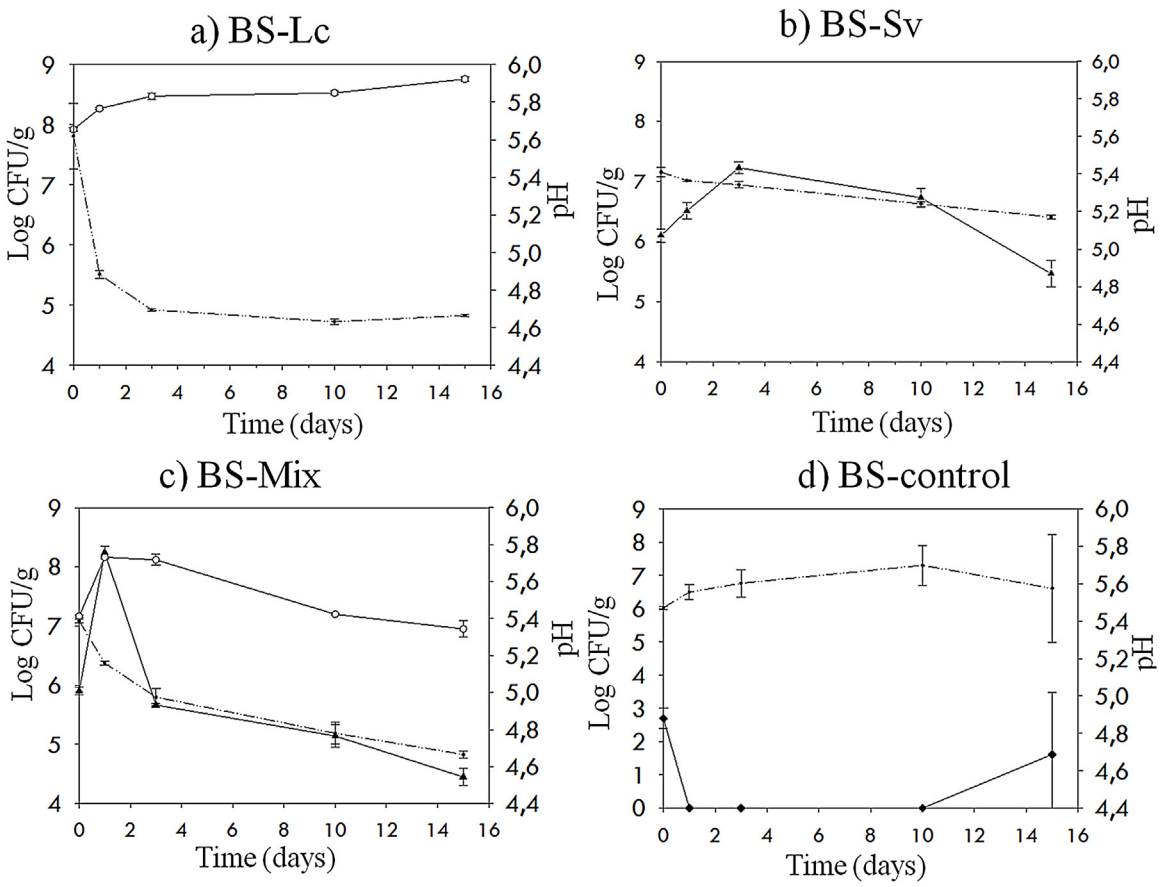

Fig. 1 – Microbial counts of beaker sausage models (BS) during 15 days of fermentation and ripening. LAB (O), CNS (▲) and pH (dash and dot). (a) BS inoculated with *L. curvatus* CRL705 (BS-Lc); (b) BS inoculated with *S. vitulinus* GV318 (BS-Sv); (c) BS inoculated with the mixed autochthonous starter culture (BS-mix) and (d) non-inoculated BS (BS-control).

3.2. *Performance of selected strains in BS models*

When BS models were inoculated with pure culture of each starter bacteria and the mixed starter culture, a satisfactory growth at 22 °C was observed (Fig. 1). The batch inoculated with *L. curvatus* CRL705 (BS-Lc) showed this microorganism was able to grow from 7.92 to 8.76 log CFU/g after 15 days with a pH reduction during the first 24 h (from 5.62 to 4.90) with a final pH value of 4.66 (Fig. 1a). On the other hand, in the batch inoculated with *S. vitulinus* GV318 (BS-Sv) a growth from 6.10 to 7.23 log CFU/g at 3 days was produced with a further decrease to 5.46 log CFU/g at the end of incubation; a slight decrease of pH values (from 5.41 to 5.17) was observed throughout incubation time (Fig. 1b). In the mixed starter culture (BS-mix) model *L. curvatus* CRL705 increased its cell number in 1 log unit after 24 h of incubation, then a slow decline was observed reaching a final number of 6.95 log CFU/g at 15 days (Fig. 1c). *S. vitulinus* exhibited a maximal growth at 24 h (8.24 log CFU/g) followed by a fast drop up to 4.44 log CFU/g at 15 days (Fig. 1c). Nevertheless, in the non-inoculated batch (BS-control), the presence of antibiotic allowed a dramatic decrease of the low numbers of meat contaminants, a slight bacterial recovery was produced after 10 days; pH values were maintained between 5.45 and 5.65. Consequently, 10 days was selected as the final time for peptidomic analysis to guarantee sterility of BS-control.

3.3. *Low molecular-weight peptides released from sarcoplasmic proteins in fermented sausage models*

BS-control, BS-Lc and BS-mix at both, initial time (t_0) and after 10 days of incubation were subjected to LC–ESI-MS/MS analysis to describe fermented meat peptidome, which comprises the generated LMW peptides (<3 kDa) shown in Tables S1–S4. Data obtained from the three sausage models at 0 h were pooled as BS-t_0 since most peptides from different models overlapped at the initial time. Initially, we performed the search using both "all entries" and "mammalian" as taxonomy restriction parameter. We realize that the obtained results were comparable using the two criteria. However, the analysis time considerably increased when using the "all entries" option. This is because of the difficulty to perform searches with the option "none" (no information available about the enzyme cleavage), which greatly increases the number of peptide sequences to compare within the databases, increasing both the search time and the identity threshold, which usually results in fewer significant matches in total. For that reason, we decided to try to reduce the great complexity of the analysis by applying the "mammalia" option as taxonomy restriction parameter. Thirty peptides were identified at t_0, probably released by endogenous proteolysis during the *post-mortem* process (Table S1). After 10 days of incubation

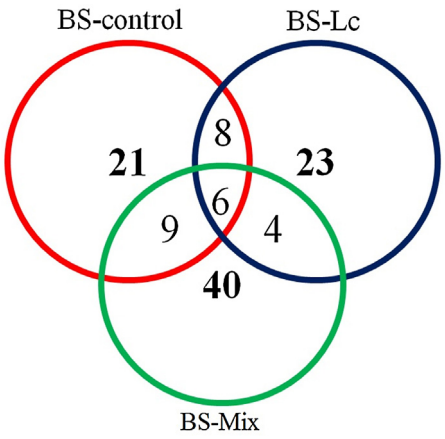

Fig. 2 – Venn diagram showing numbers of peptides obtained in the beaker sausage models at 10 days of incubation: non-inoculated BS (BS-control); BS inoculated with *L. curvatus* CRL705 (BS-Lc) and BS inoculated with the mixed autochthonous starter culture (BS-mix).

at 22 °C, in the non-inoculated batch (BS-control) the presence of 44 peptides were detected (Table S2), these findings clearly showed the activity of muscle peptidases. When the inoculated sausage models after 10 days of incubation were analyzed, 41 and 59 LMW peptides were detected from BS-Lc and BS-mix, respectively (Tables S3 and S4). Therefore, the mixed starter culture contributed to the greatest enrichment of peptides by a more intense proteolysis (59 peptides) compared to the action of endogenous enzymes (44 peptides) or *L. curvatus* CRL705 (41 peptides).

The contribution of *L. curvatus* CRL705 and the formulated starter culture on LMW peptidome changes could be elucidated by comparing the identified peptides from the three batches (BS-control, BS-Lc and BS-mix) after 10 days of incubation (Fig. 2; Tables S2–S4). Each analyzed model exhibited a different peptides variety; 23 new peptides were obtained in presence of *L. curvatus* CRL705 whereas 40 peptides, not previously identified, were detected on the sausage model inoculated with the starter culture. These results suggested that meat proteolysis during ripening was a highly dynamic process, especially when meat starter culture was present. On the other hand, a small number of peptides were common to the different models: six among the three models, eight between BS-control and BS-Lc, four between BS-Lc and BS-mix and nine between BS-control and BS-mix (Fig. 2).

Proteolysis affected a wide variety of sarcoplasmic proteins. Proteins generating the most important number of peptides were glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the BS-t_0 model; myoglobin (Mb) in BS-control, BS-Lc and BS-mix at 10 days, and fructose bi-phosphate aldolase A (ALDOA) for BS-mix model at 10 days of incubation. Hence, it could be inferred that the extent of proteolysis was strongly dependent on the incubation time as well as on the BS model involved (Fig. 3). In addition, other proteins (zinc finger protein 287, ATP citrate synthase and malate dehydrogenase, among others) produced one or few LMW peptides during incubation. Notably this is the first report evidencing the hydrolysis of these sarcoplasmic proteins group.

Concerning the proteolytic process on the above-mentioned proteins, Mb exhibited a low number of peptides at t_0 while the greatest variety of peptides was obtained at 10 days in BS-control (Fig. 3), indicating a weak degradation during the early *post-mortem* process followed by a marked activation of proteolysis throughout the ripening time. Sausage models inoculated with *L. curvatus* CRL705 and mixed starter culture, exhibited a reduced peptide diversity from Mb at the end of incubation. Concerning GAPDH, it accounted for 12 different peptides in BS-t_0, however at 10 days in the three batches (BS-control, BS-Lc and BS-mix) their diversity was reduced (Fig. 3). On the other hand, in BS-t_0 as well as BS-control and BS-Lc at 10 days, ALDOA was poorly represented by its derived LMW peptides (Fig. 3). On the contrary, when mixed starter culture was inoculated, hydrolysis of this protein allowed identifying 17 peptides at 10 days, indicating a higher peptidogenic activity of the mixed starter culture. Thus, the main target protein by the mixed starter culture was ALDOA in our experimental conditions. The activity of endogenous enzymes, without the action of the microbial peptidases, had been limited to the region 64–80 showing a remarkable action of aminopeptidases (Fig. 4; Table S4). In the presence of *L. curvatus* CRL705 (Fig. 4), a fewer number of peptides were identified, all of them were nearby the targeted region for endogenous muscle peptidases. These results sharply contrasted with the extensive degradation along the whole sequence, including 153–188 and 285–300 regions, when the mixed starter culture was inoculated. In addition, aminopeptidases action was mainly observed in the 166–188 region while carboxypeptidases were evidently active toward the 285–300 region (Fig. 4).

4. Discussion

A meat starter culture was formulated by using the bacteriocinogenic *L. curvatus* CRL705 and the selected CNS strain. Based on the susceptibility to all assayed antibiotics, lack of biogenic amines production and growth support in co-culture with *L. curvatus* CRL705, CNS was selected and identified as *S. vitulinus* (previously known as *S. vitulus*). This species was regularly isolated from farm animals, milk and dairy products as well as from artisanal fermented sausages. In fact, *S. vitulinus* has been isolated from traditional Iberian dry-fermented sausages and used together with *Pediococcus acidilactici* as a competitive autochthonous starter culture [40,41]. The presence of transferable antibiotic resistance genes in CNS used as starter culture as well as the production of biogenic amines in fermented meat products has gained attention during recent years [42,43]. It is well-known the high prevalence of antibiotic resistances in CNS isolated from spontaneously fermented sausages [44,45]. In coincidence, in this study only one among the seven CNS strains analyzed showed to be susceptible to clinical antibiotics. On the other hand, the use of negative biogenic amine-producer strains as starter cultures for fermented sausages is a suitable strategy to limit their generation [46]. In fact, this criterion was successfully used in recent studies where different fermented meat products were analyzed [47–49].

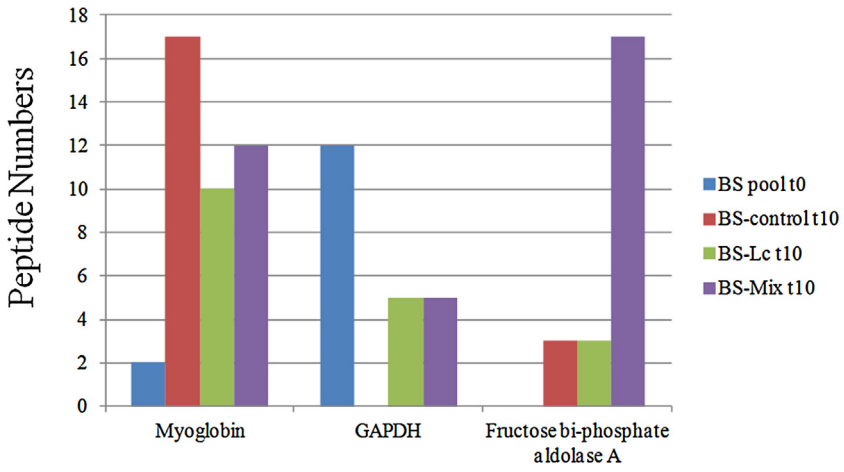

Fig. 3 – Number of peptides identified by MS/MS originated from myoglobin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and fructose-biphosphate aldolase A obtained by MS/MS for the different BS models studied: non-inoculated BS (BS-control); BS inoculated with *L. curvatus* CRL705 (BS-Lc) and BS inoculated with the mixed autochthonous starter culture (BS-mix) at 10 days of incubation at 22 °C; BS-t_0: pool of the 0 h samples corresponding to the different BS models assayed in this work.

The growth performance of both *L. curvatus* CRL705 and *S. vitulinus* GV318 during a 10-days incubation at 22 °C showed that the mixed starter culture assured pH drop of the meat batter evidencing its adaption to the beaker sausage. This result is according with those obtained from Spanish, Italian and Turkish meat products that applied *Lactobacillus* or *Pediococcus* combined with *Staphylococcus* as mixed starter cultures [23,24,50]. When growing alone in the BS-Sv model (Fig. 1b), *S. vitulinus* exhibited a steady growing phase similar to those of *S. xylosus* and *S. carnosus* strains inoculated as starter in meat products [23,51]. However, the well-known sensitivity of this microbial group to acid environment explain the remarkable decrease observed for *S. vitulinus* GV318 counts after 24 h in the mixed starter culture (Fig. 1c), this being in agreement with the results obtained with other *Staphylococcus* species used in mixed starter cultures [23,41,52]. However, the interaction between LAB and CNS species during sausage fermentation/ripening regulate flavor compounds generation, which are mostly produced by CNS microbiota [52]; nitrates reduction is also guaranteed by the CNS strains during the first steps of fermentation, thus assuring sausage color development. The acidogenic ability of *L. curvatus* CRL705 stimulated endogenous proteolysis as was previously reported for *L. plantarum* [53]. This fact exerted a great effect on LMW peptide composition; although some influence due to starter culture peptidases action could not be discarded. The single presence of *L. curvatus* CRL705, mainly enhanced the endogenous proteolysis already observed in non-inoculated BS. In addition,

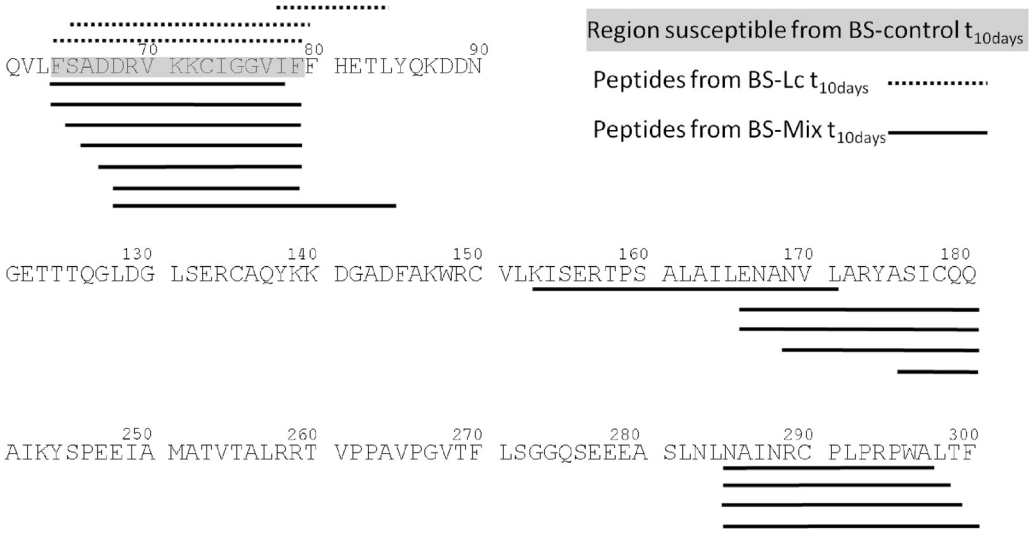

Fig. 4 – Peptide map of fructose bisphosphate aldolase A obtained in the different sausage models. BS-control (beaker sausage non-inoculated); BS-Lc (BS inoculated with *L. curvatus* CRL705); BS-mix (BS inoculated with the mixed autochthonous starter culture) at 10 days.

Table 1 – Peptides sequences originated from creatine kinase M type (CK) obtained from the analyzed models with scores >25. (*) Results from the different models at initial time (BS-control, BS-Lc and BS-mix) were pooled due to their similarities. Position: peptide localization into CK sequence (start–end).

Model	Sequence	Position
BS-t_0*	DKETPSGFTLDDVIQTGVDNPGHPF	44–68
	TLDDVIQTGVDNPGHPF	52–68
	DDVIQTGVDNPGHPF	54–68
	DDVIQTGVDNPGHPFI	54–69
	DDVIQTGVDNPGHPFIMT	54–71
	DVIQTGVDNPGHPFI	55–69
	VAGDEESYTVFKDL	75–88
	SMTEQEQQQLIDDHFL	178–193
	TEQEQQQLIDDHFL	180–193
	EQQQLIDDHFL	183–193
BS-control t_{10}	DDVIQTGVDNPGHPF	54–68
	DVIQTGVDNPGHPF	55–68
	VAGDEESYTVFKDL	75–88
	AGDEESYTVFKDL	76–88
	DVSNADRLGSSEVEQVQL	335–352
BS-Lc t_{10}	DDVIQTGVDNPGHPF	54–68
	DDVIQTGVDNPGHPFIMT	54–71
	DVIQTGVDNPGHPF	55–68
	DVIQTGVDNPGHPFI	55–69
	DVIQTGVDNPGHPFIMT	55–71
	VGCVAGDEESYTVFKDL	72–88
	VAGDEESYTVFKDL	75–88
	DVSNADRLGSSEVEQVQL	335–352
BS-Mix t_{10}	DDVIQTGVDNPGHPF	54–68
	DDVIQTGVDNPGHPFIM	54–70
	DDVIQTGVDNPGHPFIMT	54–71
	DVIQTGVDNPGHPFI	55–69
	DVIQTGVDNPGHPFIM	55–70
	DVIQTGVDNPGHPFIMT	55–71
	DVIQTGVDNPGHPFIMTVG	55–73
	VIQTGVDNPGHPFIMT	56–71
	TGEFKGKYYPLKSMTEQEQQQLIDDHFL	166–193
	DVSNADRLGSSEVEQVQL	335–352

the greatest peptide diversity was generated when mixed starter culture (BS-mix) was present in the beaker sausage model. *S. vitulinus* proteases may be involved in peptide generation since an important activity toward meat proteins was reported for CNS [16,54]; further studies may be conducted to characterize the proteolytic system of *S. vitulinus* GV318.

The production of LMW peptides because of protein degradation during incubation of each BS model was variable, and particularly affected certain proteins. In this study, Mb experienced an intense proteolysis during incubation of beaker sausages models. On the contrary, a lack of Mb hydrolysis was reported in pork meat during *post mortem* storage when two-dimentional electrophoresis (2DE) was used [55]. This difference may be assigned to the different proteomic technology herein applied. Nevertheless, a high number of peptides derived from Mb was also identified during ripening of fermented products [13,17,56] as well as in dry cured ham [57] with or without bacteria, respectively.

Furthermore, a wide range of peptides arisen from CK was detected at initial time on the different beaker sausage models (Table 1). Accordingly, an important number of peptides from CK in dry cured ham [58] as well as in meat during *post mortem* period was reported [55,59]. On the other hand, GAPDH also originated many peptides particularly at t_0 (Fig. 3); on

the contrary, its hydrolysis has been poorly found during *post mortem* period in bovine meat [59]. The lower peptide diversity in the inoculated beaker sausage models (BS-Lc and BS-mix) for Mb and GAPDH would indicate a stronger proteolysis that promoted the complete peptide degradation with the concomitant amino acid release (Fig. 3). These proteins showed a noticeable proteolysis in both fermented and non-fermented meats [21,60–62]. Based on these findings, the endogenous proteolytic system may be assigned as the main responsible for the hydrolysis of Mb, CK and GADPH. By contrast, proteolysis of ALDOA may be clearly attributed to the mixed starter culture, since only a small number of peptides were generated in the non-inoculated model (BS-control) or in the presence of *L. curvatus* CRL705 at 10 days of incubation. A preference of peptidases from *S. vitulinus* GV318 for this protein may be suggested; and the presence of new cleavage sites may also be assigned to *S. vitulinus* proteolytic system (Fig. 4). The enhancement of peptides generation in the sausage model inoculated with the mixed starter culture is in accordance to other authors. Picariello et al. [61] described the disappearance of ALDOA protein in dry fermented sausages by using 2DE, but not in dry cured hams. Thus, this protein remained as the most affected by the mixed starter culture. Moreover, proteases of *S. vitulinus* GV318 might have been able to generate cleavage sites along the whole protein sequence. However, further studies are needed to clarify if some of those herein identified peptides were exclusively originated by *S. vitulinus* GV318 or by a cooperative action of the mixed starter culture. Therefore, these new peptides could be proposed as biomarker candidates to differentiate a particular sausage manufacturing process (i.e. specific starter culture utilization). These generated peptide biomarker candidates may be further proposed as a tool for product authentication and as quality standards to distinguish PDO (Protected Designation of Origin) and PGI (Protected Geographic Indication) meat products. As the peptide fraction < 3 kDa is frequently related with flavor of raw and processed meat [14,30,63], the herein identified peptides could possibly exert some influence on flavor development.

5. Conclusion

The starter culture constituted by *L. curvatus* CRL705 and *S. vitulinus* GV318 assayed in this work, exhibited a good performance during the fermentation of beaker sausage models. *L. curvatus* have known acidogenic and bacteriocinogenic abilities, whereas *S. vitulinus* lack such factors, which may imply a safety risk. In addition, these strains generated a specific pattern of protein hydrolysis with the generation of LMW peptides. Consequently, they might guarantee hygienic and typicity characteristics of meat fermented products. The use of autochthonous functional cultures is an effective strategy to limit unsafe compounds production in traditional sausages while preserving their sensory quality. On the other hand, the identification of new peptides as well as new target proteins represents a significant step toward elucidating of the role of microorganisms in meat proteolysis. In addition, these peptides may be used, in the future, as biomarkers for product authenticity and quality. Nonetheless, their real impact on meat fermented products remains to be established.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgements

This research has been supported by grants from CONICET (PIP2011-0100406) and SECyT (PICT 0655; PICT2011 0175). In addition financial support of project AGL2012-32146 from the Spanish Ministry of Economy and Competitiveness in fully acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.euprot.2015.05.001.

REFERENCES

[1] Fontana C, Cocconcelli PS, Vignolo G. Monitoring the bacterial population dynamics during fermentation of artisanal Argentinean sausages. Int J Food Microbiol 2005;103:131–42.
[2] Fontana C, Vignolo G, Cocconcelli PS. PCR–DGGE analysis for the identification of microbial populations from Argentinean dry fermented sausages. J Microbiol Methods 2005;63:254–63.
[3] Sanz Y, Toldrá F. Purification and characterization of an arginine aminopeptidase from *Lactobacillus sakei*. Appl Environ Microbiol 2002;68:1980–7.
[4] Hugas M, Monfort JM. Bacterial starter cultures for meat fermentation. Food Chem 1997;59:547–54.
[5] Lücke F-K. Fermented sausages. In: Wood BJB, editor. Microbiology of fermented foods. 2 ed. London: Blackie Academic & Professional; 1998. p. 441–83.
[6] Lücke FK. Utilization of microbes to process and preserve meat. Meat Sci 2000;56:105–15.
[7] Hüfner E, Hertel C. Improvement of raw sausage fermentation by stress-conditioning of the starter organism *Lactobacillus sakei*. Curr Microbiol 2008;57:490–6.
[8] Talon R, Leroy S, Lebert I, Giammarinaro P, Chacornac J-P, Latorre-Moratalla M, et al. Safety improvement and preservation of typical sensory qualities of traditional dry fermented sausages using autochthonous starter cultures. Int J Food Microbiol 2008;126:227–34.
[9] Cocconcelli PS, Fontana C. Starter cultures for meat fermentation. In: Toldrá F, editor. Handbook of meat processing. Ames: Blackwell; 2010. p. 199–218.
[10] Leroy F, Verluyten J, De Vuyst L. Functional meat starter cultures for improved sausage fermentation. Int J Food Microbiol 2006;106:270–85.
[11] Ammor MS, Mayo B. Selection criteria for lactic acid bacteria to be used as functional starter cultures in dry sausage production: an update. Meat Sci 2007;76:138–46.
[12] Flores M, Toldrá F. Microbial enzymatic activities for improved fermented meats. Trends Food Sci Technol 2011;22:81–90.
[13] Fadda S, López C, Vignolo G. Role of lactic acid bacteria during meat conditioning and fermentation: peptides generated as sensorial and hygienic biomarkers. Meat Sci 2010;86:66–79.

[14] Nishimura T. Influence of the peptides produced during post mortem conditioning on improvement of meat flavor. Res Adv Qual Meat Meat Prod 2002;6:5–78.
[15] Spaziani M, Torre MD, Stecchini ML. Changes of physicochemical, microbiological, and textural properties during ripening of Italian low-acid sausages. Proteolysis, sensory and volatile profiles. Meat Sci 2009;81:77–85.
[16] Mauriello G, Casaburi A, Villani F. Proteolytic activity of *Staphylococcus xylosus* strains on pork myofibrillar and sarcoplasmic proteins and use of selected strains in the production of 'Naples type' salami. J Appl Microbiol 2002;92:482–90.
[17] Hughes MC, Kerry JP, Arendt EK, Kenneally PM, McSweeney PLH, O'Neill EE. Characterization of proteolysis during the ripening of semi-dry fermented sausages. Meat Sci 2002;62:205–16.
[19] Casaburi A, Blaiotta G, Mauriello G, Pepe O, Villani F. Technological activities of *Staphylococcus carnosus* and *Staphylococcus simulans* strains isolated from fermented sausages. Meat Sci 2005;71:643–50.
[20] Fadda S, Sanz Y, Vignolo G, Aristoy MC, Oliver G, Toldra F. Characterization of muscle sarcoplasmic and myofibrillar protein hydrolysis caused by *Lactobacillus plantarum*. Appl Environ Microbiol 1999;65:3540–6.
[21] Fadda S, Sanz Y, Vignolo G, Aristoy MC, Oliver G, Toldrá F. Hydrolysis of pork muscle sarcoplasmic proteins by *Lactobacillus curvatus* and *Lactobacillus sake*. Appl Environ Microbiol 1999;65:578–84.
[22] Sanz Y, Sentandreu MA, Toldrá F. Role of muscle and bacterial exopeptidases in meat fermentation. Res Adv Qual Meat Meat Prod 2002;14:3–55.
[23] Casaburi A, Di Monaco R, Cavella S, Toldrá F, Ercolini D, Villani F. Proteolytic and lipolytic starter cultures and their effect on traditional fermented sausages ripening and sensory traits. Food Microbiol 2008;25:335–47.
[24] Aro Aro JM, Nyam-Osor P, Tsuji K, Shimada K.-I., Fukushima M, Sekikawa M. The effect of starter cultures on proteolytic changes and amino acid content in fermented sausages. Food Chem 2010;119:279–85.
[25] Lametsch R, Karlsson A, Rosenvold K, Andersen HJ, Roepstorff P, Bendixen E. Postmortem proteome changes of porcine muscle related to tenderness. J Agric Food Chem 2003;51:6992–7.
[26] Soloviev M. Peptidomics divide et impera. In: Soloviev M, editor. Peptidomics. New York (USA): Humana Press; 2010. p. 3–9.
[27] Picariello G, Mamone G, Addeo F, Ferranti P. Novel mass spectrometry-based applications of the 'omic' sciences in food technology and biotechnology. Food Technol Biotechnol 2012;50:286–305.
[28] Lahrichi SL, Afolter M, Zolezzi IS. Food peptidomics large scale analysis of small bioactive peptides – a pilot study. J Proteomics 2013;88:83–91.
[29] Schägger H, Von Jagow G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 1987;166:368–79.
[30] Sentandreu MA, Stoeva S, Aristoy MC, Laib K, Voelter W, Toldrá F. Identification of small peptides generated in Spanish dry-cured ham. J Food Sci 2003;68:64–9.
[31] Sentandreu MA, Sentandreu E. Peptide biomarkers as a way to determine meat authenticity. Meat Sci 2011;89:280–5.
[32] Mora L, Sentandreu MA, Koistinen KM, Fraser PD, Toldrá F, Bramley PM. Naturally generated small peptides derived from myofibrillar proteins in serrano dry-cured ham. J Agric Food Chem 2009;57:3228–34.
[33] Vignolo GM, Suriani F, Holgado APdR, Oliver G. Antibacterial activity of *Lactobacillus strains* isolated from dry fermented sausages. J Appl Microbiol 1993;75:344–9.
[34] Vignolo G, Fadda S, De Kairuz MN, De Holgado APR, Oliver G. Effects of curing additives on the control of *Listeria monocytogenes* by lactocin 705 in meat slurry. Food Microbiol 1998;15:259–64.
[35] Castellano PH, Holzapfel WH, Vignolo GM. The control of *Listeria innocua* and *Lactobacillus sakei* in broth and meat slurry with the bacteriocinogenic strain *Lactobacillus casei* CRL705. Food Microbiol 2004;21:291–8.
[36] Vignolo GM, de Ruiz Holgado AAP, Oliver G. Isolation and identification of the Lactic Acid Bacteria involved in the ripening of cured meat products. Microbiol Aliment Nutr 1986;4:87–94.
[37] Vignolo G, Ruiz Holgado AP, Oliver G. Isolation and identification of the lactic acid bacteria involved in the ripening of the cured meat products. Microbiol Aliment Nutr 1986;4:87–94.
[38] Schillinger U, Lücke FK. Antibacterial activity of *Lactobacillus sake* isolated from meat. Appl Environ Microbiol 1989;55:1901–6.
[39] Fadda S, Vignolo G, Oliver G. Tyramine degradation and tyramine/histamine production by lactic acid bacteria and *Kocuria* strains. Biotechnol Lett 2001;23:2015–9.
[40] Benito MJ, Martín A, Aranda E, Pérez-Nevado F, Ruiz-Moyano S, Córdoba MG. Characterization and selection of autochthonous lactic acid bacteria isolated from traditional Iberian dry-fermented salchichón and chorizo sausages. J Food Sci 2007;72:M193–201.
[41] Casquete R, Benito MJ, Martín A, Ruiz-Moyano S, Hernández A, Córdoba MG. Effect of autochthonous starter cultures in the production of salchichón, a traditional Iberian dry-fermented sausage, with different ripening processes. LWT – Food Sci Technol 2011;44:1562–71.
[42] Kastner S, Perreten V, Bleuler H, Hugenschmidt G, Lacroix C, Meile L. Antibiotic susceptibility patterns and resistance genes of starter cultures and probiotic bacteria used in food. Syst Appl Microbiol 2006;29:145–55.
[43] Talon R, Leroy S. Diversity and safety hazards of bacteria involved in meat fermentations. Meat Sci 2011;89:303–9.
[44] Even S, Leroy S, Charlier C, Ben Zakour N, Chacornac JP, Lebert I, et al. Low occurrence of safety hazards in coagulase negative staphylococci isolated from fermented foodstuffs. Int J Food Microbiol 2010;139:87–95.
[45] Marty E, Bodenmann C, Buchs J, Hadorn R, Eugster-Meier E, Lacroix C, et al. Prevalence of antibiotic resistance in coagulase-negative staphylococci from spontaneously fermented meat products and safety assessment for new starters. Int J Food Microbiol 2012;159:74–83.
[46] Bover-Cid S, Izquierdo-Pulido M, Vidal-Carou MC. Effect of the interaction between a low tyramine-producing *Lactobacillus* and proteolytic staphylococci on biogenic amine production during ripening and storage of dry sausages. Int J Food Microbiol 2001;65:113–23.
[47] Casquete R, Benito MJ, Martín A, Ruiz-Moyano S, Pérez-Nevado F, Córdoba MG. Comparison of the effects of a commercial and an autochthonous *Pediococcus acidilactici* and *Staphylococcus vitulus* starter culture on the sensory and safety properties of a traditional Iberian dry-fermented sausage salchichón. Int J Food Sci Technol 2012;47:1011–9.
[48] Mangia NP, Trani A, Di Luccia A, Faccia M, Gambacorta G, Fancello F, et al. Effect of the use of autochthonous *Lactobacillus curvatus*, *Lactobacillus plantarum* and *Staphylococcus xylosus* strains on microbiological and biochemical properties of the Sardinian fermented sausage. Eur Food Res Technol 2013;236:557–66.
[49] Ciuciu Simion AM, Vizireanu C, Alexe P, Franco I, Carballo J. Effect of the use of selected starter cultures on some quality, safety and sensorial properties of Dacia sausage, a traditional Romanian dry-sausage variety. Food Control 2014;35:123–31.

[50] Dalmış Ü, Soyer A. Effect of processing methods and starter culture (*Staphylococcus xylosus* and *Pediococcus pentosaceus*) on proteolytic changes in Turkish sausages (sucuk) during ripening and storage. Meat Sci 2008;80:345–54.
[51] Bover-Cid S, Izquierdo-Pulido M, Vidal-Carou MC. Effect of proteolytic starter cultures of *Staphylococcus* spp. on biogenic amine formation during the ripening of dry fermented sausages. Int J Food Microbiol 1999;46:95–104.
[52] Ravyts F, Steen L, Goemaere O, Paelinck H, De Vuyst L, Leroy F. The application of staphylococci with flavour-generating potential is affected by acidification in fermented dry sausages. Food Microbiol 2010;27:945–54.
[53] Fadda S, Vildoza MJ, Vignolo G. The acidogenic metabolism of *Lactobacillus plantarum* CRL 681 improves sarcoplasmic protein hydrolysis during meat fermentation. J Muscle Foods 2010;21:545–56.
[54] Mauriello G, Casaburi A, Blaiotta G, Villani F. Isolation and technological properties of coagulase negative staphylococci from fermented sausages of Southern Italy. Meat Sci 2004;67:149–58.
[55] Lametsch R, Roepstorff P, Bendixen E. Identification of protein degradation during post-mortem storage of pig meat. J Agric Food Chem 2002;50:5508–12.
[56] Candogan K, Wardlaw FB, Acton JC. Effect of starter culture on proteolytic changes during processing of fermented beef sausages. Food Chem 2009;116:731–7.
[57] Mora L, Toldrá F. Proteomic identification of small (<2000 Da) myoglobin peptides generated in dry-cured ham. Food Technol Biotechnol 2012;50:343–9.
[58] Mora L, Sentandreu MA, Fraser PD, Toldrá F, Bramley PM. Oligopeptides arising from the degradation of creatine kinase in Spanish dry-cured ham. J Agric Food Chem 2009;57:8982–8.
[59] Stoeva S, Byrne CE, Mullen AM, Troy DJ, Voelter W. Isolation and identification of proteolytic fragments from TCA soluble extracts of bovine *M. longissimus dorsi*. Food Chem 2000;69:365–70.
[60] Di Luccia A, Picariello G, Cacace G, Scaloni A, Faccia M, Liuzzi V, et al. Proteomic analysis of water soluble and myofibrillar protein changes occurring in dry-cured hams. Meat Sci 2005;69:479–91.
[61] Picariello G, De Martino A, Mamone G, Ferranti P, Addeo F, Faccia M, et al. Proteomic study of muscle sarcoplasmic proteins using AUT-PAGE/SDS-PAGE as two-dimensional gel electrophoresis. J Chromatogr B 2006;833:101–8.
[62] Sanz Y, Fadda S, Vignolo G, Aristoy MC, Oliver G, Toldrá F. Hydrolytic action of *Lactobacillus casei* CRL 705 on pork muscle sarcoplasmic and myofibrillar proteins. J Agric Food Chem 1999;47:3441–8.
[63] Henriksen AP, Stahnke LH. Sensory and chromatographic evaluations of water soluble fractions from dried sausages. J Agric Food Chem 1997;45:2679–84.